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(54) GENE IMPARTING FLOCCULABILITY TO YEAST AND GENE PRODUCT

(57) The present invention relates to a protein having an activity of conferring on yeast brewer's yeast-type flocculating property; a DNA coding for the protein; a plasmid containing the DNA; a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been conferred or enhanced with the use of the DNA and a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been eliminated or reduced with the use of the DNA; and a method for eliminating or reducing brewer's yeast-type flocculating property of yeast by inhibiting the expression of the DNA.

The present invention also relates to a yeast strain wherein brewer's yeast-type flocculating property has been conferred, enhanced, eliminated or reduced by the above method.

Further, the present invention relates to a method for producing a brewed product comprising culturing the above yeast strain as well as a brewed product obtained by the method.

According to the present invention, there are provided the Lg-Flo1 protein having an activity of conferring on yeast brewer's yeast-type flocculating property and the DNA strand of Lg- *FLO1* coding for the protein. By introducing the above DNA of the invention into yeast cells as a foreign gene by genetic engineering techniques, i.e., introducing the DNA into yeast cells as an extranuclear gene and(/or) a nuclear gene, it is possible to confer on yeast brewer's yeast-type flocculating property or enhance this property in yeast. On the contrary, by introducing a DNA is obtained by destroying the DNA of the invention into yeast cells, or by inhibiting the expression of the DNA of the invention, it is possible to convert a flocculent yeast strain into a non-flocculent strain, or to reduce flocculating property.

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Description

Field of the Invention

The present invention relates to a yeast flocculation gene and the use thereof. More specifically, the present invention relates to a protein having an activity of conferring on yeast brewer's yeast-type flocculating property, a DNA coding for the protein, a plasmid containing the DNA, a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been conferred or enhanced with the use of the DNA, a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been eliminated or reduced with the use of the DNA, and a method for eliminating or reducing brewer's yeast-type flocculating property of yeast by inhibiting the expression of the DNA.

The present invention also relates to a yeast strain wherein brewer's yeast-type flocculating property has been conferred, enhanced, eliminated or reduced by one of the above-mentioned methods.

Further, the present invention relates to a method for producing brewed products comprising culturing the above yeast strain as well as brewed products obtained by the method.

Background Art

It is a well known fact that, in alcoholic drinks such as beer and wine, the flocculating property of yeast used in fermentation is important not only because it determines the flavor and taste of the resultant products but also because it influences the workability in the fermentation process. The yeast which is used in the brewing of lager beer widely produced in Germany, Japan and many other countries has a characteristic that cells flocculate and sediment to the bottom of the fermented wort near the end of fermentation; such yeast is particularly called bottom fermenting yeast. In beer brewing, there is a characteristic feature in the process not found in other brewing, that is, yeast cells sedimented at the end of fermentation are recovered and reused in the subsequent fermentation. Accordingly, the characteristic of bottom fermenting yeast that it sediments at the late stage of fermentation has an especially big meaning for beer brewing.

Since brewer's yeast is one of the major factors that determine the quality of the final beer product, the breeding of excellent yeast strains is an important subject for beer brewers. In the breeding of bottom fermenting yeast, the conferring of a suitable flocculating property on yeast has a significant meaning. This is because a yeast strain too strong in flocculating property precipitates in the fermented wort during the fermentation, which leads to the termination of the fermentation, and, on the other hand, a yeast strain lacking flocculating property remains suspending even at the late stage of the fermentation and an operation such as centrifugation is needed to remove yeast cells from fermented wort. Therefore, a desirable yeast strain for the current production method is a strain which is dispersed at the beginning of the fermentation and precipitates well at the late stage of the fermentation. If the production method is different, needless to say, a yeast strain having a flocculating property suitable for the method is required.

In spite of the numerous studies concerning the yeast flocculating property which is industrially significant, the mechanism of yeast flocculation has not been elucidated yet. It is hard to say that control of flocculating property by improving a yeast strain *per se* is successful. As a result of years of genetic researches, the existence of yeast genes such as *FLO1*, *flo3*, *FLO5*, *FLO8*, *sfl1*, *fsu1*, *fsu2*, *tup1*, *cyc8*, *cka2*, *FMC1* as well as the genes *oli1* and *oxi2* in mitochondrial DNA have been confirmed as genes involved in the flocculating property of yeast. As a study of these genes involved in the flocculating property of yeast at the molecular level, the isolation and analysis of the *FLO1* gene has been performed [Yeast, 9, 423 (1993) and Yeast, 10, 211 (1994)]. Also, the isolation and analysis of the *FLO5* gene has been reported. This report has shown that, although the location of the *FLO5* gene is different from that of the previously reported *FLO1* gene in the yeast chromosomal DNA, the restriction map and nucleotide sequence of *FLO5* gene are almost identical with those of *FLO1* [J. Inst. Brew., 85, 95, (1979) and Curr. Genet., 25, 196 (1994)].

However, the analysis of these genes at the molecular level have not been sufficient and it has not been elucidated yet in what mechanism these genes are involved in the flocculating property of yeast. Furthermore, with respect to genes other than *FLO1* and *FLO5* involved in the yeast flocculating property, even isolation or structural analysis has not been performed. No report has been made on the proteins which these genes code for.

As an attempt to improve the flocculating property of yeast by using such a gene as described above involved in that property, there has been a report in which flocculating property was conferred on various non-flocculent yeast strains including brewer's yeast by introducing the *FLO1* gene, a flocculation gene of *Saccharomyces cerevisiae* [Agric. Biol. Chem., 55, 1547 (1991)]. However, it has been reported that the flocculating ability of the thus obtained transformed brewer's yeast is expressed from the initial stage of fermentation and that fermentation tends to be delayed (Journal of the Brewing Society of Japan, 88, 665 (1993)]. Thus, the conferring of flocculating property on yeast with this *FLO1* gene cannot be said to be controlled in a favorable mode, and further improvement has been required in order to put such a method into actual use. Furthermore, although *FLO1* gene has been known to be able to confer on yeast flocculating property, the role of its gene product in yeast flocculation has not been elucidated. As a result of the analysis of the amino acid sequence deduced from the nucleotide sequence for *FLO1* gene, it has been presumed that

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the *FLO1* gene product is localized in the surface layer of yeast cells. It seems that this presumption is also supported by a report that the amino acid sequence of the N-terminal 14 residues of a protein (flocculin) obtainable specifically from the surface layer of flocculent brewer's yeast cells has some homology to the amino acid sequence presumed from the nucleotide sequence for *FLO1* gene [Appl. Environ. Microbiol., 60, 2754 (1994)], but the mechanism of the above protein has not been elucidated yet. Consequently, the attempt to control yeast flocculation by the *FLO1* gene has reached its limits.

As an attempt to use a gene other than *FLO1*, the conferring of a hereditary character on yeast was tried using the *FLO5* gene and the cell fusion method, and the utility of conferring of the hereditary character has been shown [J. Inst. Brew., 98, 315 (1992)]. However, since the method of introducing a hereditary character is the cell fusion method, it was difficult to obtain a yeast strain having the character of interest and, furthermore, there has arisen a problem that DNA sequences other than the flocculation-related gene of interest are introduced into the resultant yeast strain; e.g., the *POF1* gene which adds the phenolic flavor to beer and which many strains of *Saccharomyces cerevisiae* have is introduced simultaneously [Proc. Eur. Brew. Conv. 497 (1981)]. Thus, the improvement of flocculating property of useful yeast strains by this method cannot be said to be controlled.

As so far described, the improvements of the flocculation ability of yeast using those genes involved in yeast flocculation which have been attempted to date are of no practical use.

Under circumstances, the present invention aims at the following matters:

(1) to provide a protein having an activity of conferring on yeast brewer's yeast-type flocculating property:
(2) to provide a DNA strand coding for a protein having an activity of conferring on yeast brewer's yeast-type flocculating property:
(3) to provide a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been conferred or enhanced with the use of the above DNA strand, or a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been eliminated or reduced with the use of the above DNA strand, and a yeast strain wherein brewer's yeast-type flocculating property has been conferred, enhanced, eliminated or reduced by the above-mentioned method;
(4) to provide a method for eliminating or reducing brewer's yeast-type flocculating property of a yeast strain by inhibiting the expression of the above DNA strand; and
(5) to provide also a method for producing brewed products comprising culturing the yeast strain described above as well as brewed products obtained by the method.

Disclosure of the Invention

Toward the solution of the problems described above, the present inventors have made intensive and extensive researches into the flocculating property of bottom fermenting brewer's yeast. As a result, the inventors confirmed the existence of the *FLO1* -homologous gene which flocculent bottom fermenting yeast specifically has (hereinafter referred to as the "Lg-*FLO1* (gene)"), and elucidated the relationship between the Lg- *FLO1* gene and flocculating property. Subsequently, the inventors allowed the Lg-*FLO1* gene product to be produced in a yeast strain which had become non-flocculent due to the disraption of *FLO1* caused by the introduction of Lg-*FLO1*. As a result, the inventors have found that brewer's yeast-type flocculation can be induced by the introduction of Lg-*FLO1* gene. This finding implies not only the conferring of brewer's yeast-type flocculating property but also the conversion of a yeast strain having an laboratory yeast-type flocculating property into a yeast strain having brewer's yeast-type flocculating property. Further, the inventors have determined the region of the Lg-*FLO1* gene product which controls the brewer's yeast-type flocculation in yeast. Also, the inventors have succeeded in converting a flocculent, brewer's yeast strain into a non-flocculent strain by introducing thereinto a disrupted Lg- *FLO1* gene. Thus, the present invention has been achieved.

The present invention provides the Lg-*FLO1* gene product having the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing; or a protein which comprises a peptide having the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing and which has an activity of conferring on yeast brewer's yeast-type flocculating property: or a protein which comprises a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing the amino acid residues from position 25 to position 213 and which has an activity of conferring on yeast brewer's yeast-type flocculating property: or a protein which comprises a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing at least the amino acid residues from position 25 to position 97 and which has an activity of conferring on yeast brewer's yeast-type flocculating property: and a polypeptide which has an activity of conferring on yeast brewer's yeast-type flocculating property and which has the amino acid sequence shown substantially in SEQ ID NO: 2 in the Sequence Listing. The term "substantially" used herein means that the amino acid sequence may have deletion, substitution, addition, polymerization and the like of one or several amino acid residues as long as the amino acid sequence has an activity of conferring on yeast brewer's yeast-type flocculating property.

Also, the present invention provides a DNA strand comprising a nucleotide sequence coding for the amino acid

sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing, or a DNA comprising a nucleotide sequence coding for a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing the amino acid residues from position 25 to position 213 ; or a DNA comprising a nucleotide sequence coding for a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing at least the amino acid residues from position 25 to position 97. The term "substantially" used herein means that the amino acid sequence may have deletion, substitution, addition, polymerization and the like of one or several amino acid residues as long as the amino acid sequence has an activity of conferring on yeast brewer's yeast-type flocculating property.

Further, the present invention provides a DNA which comprises a nucleotide sequence coding for an amino acid sequence having an activity of conferring on yeast brewer's yeast-type flocculating property and which comprises a partial sequence of the nucleotide sequence shown in SEQ ID NO: 3 in the Sequence Listing containing the nucleotide sequence from position 59 to position 697 or a DNA complementary thereto; a DNA which comprises a nucleotide sequence coding for a protein having an activity of conferring on yeast brewer's yeast-type flocculating property and which comprises a partial sequence of the nucleotide sequence shown in SEQ ID NO: 3 in the Sequence Listing containing the nucleotide sequence from position 131 to position 697 or a DNA complementary thereto; a DNA comprising a partial sequence of the nucleotide sequence shown in SEQ ID NO: 3 in the Sequence Listing containing the nucleotide sequence from position 131 to position 349 or a DNA complementary thereto; and a DNA which comprises the nucleotide sequence shown in SEQ ID NO: 4 in the Sequence Listing and which codes for a polypeptide having an activity of conferring on yeast brewer's yeast-type flocculating property or a DNA complementary thereto.

The present invention also provides a DNA which is incorporated into plasmid KTYT2, YESKT2, KNWtC3 or KNYES and which comprises a nucleotide sequence coding for a protein having an activity of conferring on yeast brewer's yeast-type flocculating property as well as a plasmid comprising the above DNA.

Further, the present invention provides a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been conferred or enhanced, characterized by introducing thereinto the above-mentioned DNA. The invention also provides a method for producing a yeast strain wherein brewer's yeast-type flocculating property has been eliminated or reduced, characterized by introducing thereinto a DNA of which the ability to express a protein having an activity of conferring brewer's yeast-type flocculating property has been eliminated or reduced by disrupting the above-mentioned DNA.

Still further, the present invention provides a yeast strain which is produced by any one of the above-mentioned methods and in which brewer's yeast-type flocculating property has been conferred, enhanced, eliminated or reduced.

The present invention also provides a method for eliminating or reducing brewer's yeast-type flocculating property of yeast by inhibiting the expression of the above-mentioned DNA.

Further, the present invention provides a method for producing brewed products comprising culturing the yeast strain described above as well as brewed products obtained by the method.

Brief Description of the Drawings

Fig. 1 shows the results (photographs) of electrophoresis in Southern and Northern analyses of a brewer's yeast strain and meiotic segregants thereof.

Fig. 2 shows a restriction map of the Lg-*FLO1* gene.

Fig. 3 shows a concept diagram of the full length cloning of the Lg-*FLO1* gene by inverse PCR.

Fig. 4 shows a restriction map of the Lg-*FLO1* gene full length fragment.

Fig. 5 is a concept diagram showing the construction of an Lg-Sc-chimeric *FLO1* gene.

Fig. 6 is a chart showing the construction of a plasmid for destroying the Lg-*FLO1* gene.

Fig. 7 is a chart (continued) showing the construction of a plasmid for disrupting the Lg-*FLO1* gene.

Fig. 8 shows comparison of the deduced amino acid sequences for N-terminal portions of the Lg-*FLO1* gene and the Sc-*FLO1* gene, respectively.

Fig. 9 shows the phenotypes of flocculation in those strains having various modified *FLO1* genes.

Best Mode for Carrying Out the Invention

Hereinbelow, the present invention will be described in detail.

It should be noted that the terms "DNA", "nucleotide sequence", " gene" and "DNA strand" are used herein as having substantially the same meaning. Also, it should be noted that the terms "amino acid sequence", "peptide" and "protein" are used herein as having substantially the same meaning.

(Intercellular Flocculation in Yeast)

It is known that intercellular flocculation in yeast is attributable to sexual flocculation between a cells and α cells,

unseparatedness of budding daughter cells from mother cells, non-sexual flocculation and so forth Of these factors, the present invention aims at controlling non-sexual flocculation.

As a model for explaining the mechanism of non-sexual flocculation, the lectin hypothesis is strong that two neighboring yeast cells make interaction to each other through the linkage of a lectin like protein in the surface layer of flocculent yeast cells with sugar chains [J. Bacteriol., 150, 878 (1982)], but the lectin like protein has not been identified yet. This is one of the reasons for which the control of yeast flocculation is still difficult.

It has been reported that non-sexual flocculation can be roughly classified into two types depending on the kind of sugar which inhibits the flocculation, i.e., mannose-specific Flo1 type flocculation and NewFlo type flocculation which is inhibited by maltose, glucose and the like in addition to mannose [Yeast, 7, 559 (1991)]. The present inventors have found that the flocculating property of common bottom fermenting yeast is classified into the NewFlo type. For facilitating understanding, these types of flocculation properties are expressed with the following terms.

Briefly, the flocculating property of common laboratory yeasts that is inhibited by co-existing mannose but not inhibited by maltose and glucose is expressed herein with the term "laboratory yeast-type flocculating property". On the other hand, the flocculating property of those yeasts strains represented by common bottom fermenting brewer's yeast that is inhibited by maltose, glucose and the like in addition to co-existing mannose is expressed with the term "brewer's yeast-type flocculating property". Both types of flocculating properties are not inhibited by galactose. The present inventors presume it very important, at least in beer brewing, that bottom fermenting brewer's yeast has the "brewer's yeast-type flocculating property" for the reasons described below. Briefly, this "brewer's yeast-type flocculating property" is greatly different from the flocculating property of laboratory yeast in that the former is inhibited by glucose, maltose and the like. Beer is produced by brewing wort with brewer's yeast. Since about 6% of maltose and about 1% of glucose are contained in wort, the fact that the flocculation of brewer's yeast is inhibited by these sugars has an important meaning. In other words, it can be conjectured as follows. Due to the characteristic of the "brewer's yeast-type flocculating property", brewer's yeast added to wort is prevented from flocculation by sugars contained therein and able to disperse into the wort. Thus, the fermentation proceeds rapidly, and when sugar concentrations in the fermented wort decrease at the late stage of the fermentation, inhibition of flocculation becomes weakened. As a result, yeast cells form flocs and sediment to thereby make it easy to recover them.

(Lg-Flo1 Protein)

Lg-Flo1 protein is the protein encoded by the *FLO1*-homologous gene, namely the Lg-*FLO1* gene, peculiar to bottom fermenting brewer's yeasts and meiotic segregants thereof exhibiting the brewer's yeast-type flocculating property.

The present invention includes the Lg-Flo1 protein and derivatives thereof. The Lg-Flo1 protein may be derived from yeast, in particular *Saccharomyces cerevisiae* and it has a characteristic of being capable of conferring on yeast the brewer's yeast-type flocculating property. The Lg-Flo1 protein contains in its amino acid sequence the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing. "The amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing" includes, in addition to the amino acid sequence shown in SEQ ID NO: 1 in the Sequence Listing", the amino acid sequence of SEQ ID NO: 1 having modifications, i.e., having addition, insertion, deletion or substitution of one or more amino acid residues, as long as the amino acid sequence confers on yeast the brewer's yeast-type flocculating property.

"The amino acid sequence shown in SEQ ID NO: 1 in the Sequence Listing" of the invention has some homology to the amino acid sequence deduced from the known nucleotide sequence for the laboratory yeast *FLO1* gene. However, the decisive difference between the two is that the Lg-Flo1 protein and derivatives thereof comprising "the amino acid sequence shown in SEQ ID NO: 1 in the Sequence Listing" of the invention can confer on yeast the "brewer's yeast-type flocculating property" described above which is an important characteristic for brewer's yeast. Upon completion of the present invention, it has been shown for the first time that the Lg-Flo1 protein and derivatives thereof can confer on yeast the "brewer's yeast-type flocculating property".

With respect to flocculin which is a protein obtained from the surface layer of flocculent brewer's yeast cells, its biological functions have not been elucidated yet but the amino acid sequence for the 16 residues at its N-terminal has been determined (*TQACLPVG*RKNGMN: * represents an unidentified amino acid residue) [Appl. Environ. Microbiol., 60, 2754 (1994)]. The Lg-Flo1 protein of which the functions have been elucidated by the present invention for the first time comprises this amino acid sequence (from position 25 to position 40 of SEQ ID NO: 1 in the Sequence Listing). At present, there is no evidence showing that the Lg-Flo1 protein of the invention is identical with flocculin. However, in the Lg-Flo1 protein of the invention also, there is an extremely high possibility that the region represented by the amino acid sequence extending from position 1 to position 24 of SEQ ID NO: 1 is a secretion signal sequence necessary for the Lg-Flo1 protein to be localized in the surface layer of cells. Accordingly, it is conjectured that the protein which is localized in the surface layer of flocculent yeast cells and which has an activity of conferring on yeast flocculating property is a protein having the amino acid sequence covering from position 25 to the end of SEQ ID NO: 1 in the Sequence Listing.

(Lg-*FLO1* gene)

The present invention includes the DNA strand of Lg- *FLO1*. The term "the DNA strand of Lg-*FLO1*" used herein denotes a DNA comprising a nucleotide sequence coding for the Lg-Flo1 protein or a derivative thereof having an activity of conferring on yeast the brewer's yeast-type flocculating property.

More specifically, the present invention includes a DNA strand comprising a nucleotide sequence coding for a protein having the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing. Here, it should be noted that the expression "a nucleotide sequence coding for a protein having the amino acid sequence" means all the different nucleotide sequences which may be generated due to degeneracy of genetic codes.

What was indispensable for the completion of the present invention was the finding described in Example 1 that a bottom fermenting yeast strain and meiotic segregants thereof exhibiting the brewer's yeast-type flocculating property have the distinctive *FLO1* - homologous gene. In the present specification, this "*FLO1* - homologous gene distinctive of bottom fermenting yeast and meiotic segregants thereof exhibiting the brewer's yeast-type flocculating property" is expressed with the term "the Lg- *FLO1* gene". However, the above finding alone could not at all have lead to the fact that " the Lg- *FLO1* gene" has an activity to render on yeast the "brewer's yeast-type flocculating property". Further elaboration was required for the completion of the present invention.

From another point of view, the present invention also includes a DNA which is incorporated in plasmid KTYT2 and which comprises a nucleotide sequence coding for a protein having an activity to render on yeast the brewer's yeast-type flocculating property.

Hereinafter, the DNA's of the present invention as described above are collectively called "the Lg-*FLO1* DNA" strand.

The Lg-*FLO1* DNA strand of the invention may be a naturally occurring DNA, a totally synthesized DNA, or a partially synthesized DNA which has been synthesized using a part of a naturally occurring DNA.

(Transformation)

By introducing the Lg-*FLO1* gene DNA strand of the invention, it is possible to obtain a yeast strain wherein the brewer's yeast-type flocculating property has been conferred or enhanced.

As a method for introducing the Lg-*FLO1* DNA strand, standard techniques conventionally used in the field of genetic engineering may be used in accordance with conventional standards [see, e.g., Analytical Biochemistry 163, 391 (1987)]. Specific examples of such methods include, e.g., a method in which a desired DNA is incorporated into a vector, that is then introduced into yeast cells and a method in which a desired DNA is directly introduced into yeast cells without incorporation into a vector.

In the former method in which a desired DNA is incorporated into a vector, that is then introduced into yeast cells, vectors which may be used for this purpose include, for example, a YRp vector (a yeast multicopy vector using an ARS sequence of the yeast chromosome as its replication origin), a YEp vector (a yeast multicopy vector having a replication origin of yeast 2 μm DNA), a YCp vector (a yeast single copy vector having an ARS sequence of the yeast chromosome as its replication origin and also having a centromere sequence of the yeast chromosome) and a YIp vector (a vector to be integrated into the yeast chromosome, not having a replication origin of yeast); any of known vectors may be used. These vectors are disclosed in literature (see "New Biotechnology of Yeast", Medical Publication Center, p. 284) and may be readily prepared.

As a representative technique of introducing DNA directly into yeast cells without the incorporation of the DNA into a vector, the co-transformation method may be given in which yeast cells are co-transformed with a plasmid having a marker gene (such as a drug resistance gene) and a DNA sequence to be introduced (Japanese Examined Patent Publication No. 5-60918).

In such methods as described above, in order to express the introduced DNA sequence in yeast or to enhance or reduce its expression, a promoter (which is a unit controlling transcription and translation) and a terminator are incorporated into the DNA chain of the invention in a 5' upstream region and a 3' downstream region, respectively. As a promoter and a terminator for the above purposes, those derived from known genes such as alcohol dehydrogenase gene [J. Biol. Chem., 257, 3018 (1982)], phosphoglycerate kinase gene [Nucleic Acids Res., 10, 7791 (1982)], glycerolaldehyde-3-phosphodehydrogenase gene [J. Biol. Chem., 254, 9839 (1979)], or those which have been obtained by artificially improving the above ones may be used in addition to those derived from the Lg-*FLO1* gene *per se*. More specifically, promoters and terminators such as ADH (also known as ADC), GAPDH (also known as GPD), PHO, GAL, PGK, ENO, TRP and HIP may be used.

Further, by selecting an appropriate promoter, it is also possible to allow the gene having the DNA chain of the invention to be expressed in yeast cells in a controlled manner. For example, when a promoter from galactokinase gene is used, expression of the gene can be increased by changing the sugar source of a medium, for example, from glucose to galactose.

Furthermore, by introducing a DNA of which the ability to express the Lg-Flo1 protein has been eliminated or

reduced by disrupting the Lg-*FLO1* gene, it is possible to obtain a yeast strain of which the flocculating property has been eliminated or reduced. The disruption of the Lg- *FLO1* gene may be achieved by adding or deleting one or more bases in a region involved in the expression of the Lg-Flo1 protein in the Lg-*FLO1* gene (such as the promoter region or the coding region), or by deleting such a region as a whole. A DNA of which the ability to express the Lg-Flo1 protein has been eliminated or reduced by disrupting the Lg- *FLO1* gene may be introduced into yeast cells using the same technique as used in the above-mentioned DNA introduction. It is considered as follows. With the introduction of this DNA, homologous recombination occurs between the Lg- *FLO1* gene in the chromosomal DNA of the host yeast cells and the introduced DNA, and the Lg-*FLO1* gene of the host cells is interrupted. This leads to elimination or reduction of the ability to express the Lg-Flo1 protein, and, as a result, the flocculating property of the host yeast cells is eliminated or reduced.

A yeast strain to be transformed, i.e., a host yeast strain in the invention may be any which can be taxonomically classified into yeast. For the purpose of the invention, industrial yeast belonging to *Saccharomyces cerevisiae*, more specifically, brewer's yeast, wine yeast or yeast used for alcohol production is preferable.

The present invention also includes a method for eliminating or reducing the flocculating property of yeast by inhibiting the expression of the above-mentioned Lg-*FLO1* gene. Specific examples of this method include a method of introducing a DNA of which the ability to express the Lg-Flo1 protein has been eliminated or reduced by disrupting the Lg-*FLO1* gene, the antisense RNA method, and the like.

The present invention also includes a method, as described in (6) in Example 1, of converting the laboratory yeast-type flocculating property into the brewer's yeast-type flocculating property by substituting the above-mentioned Lg-*FLO1* gene with the laboratory yeast-type *FLO1* gene. Also, reverse conversion may be possible by using the Lg-*FLO1* gene DNA provided by the invention.

Brewed products produced by a process comprising culturing the yeast strain of the invention include alcoholic drinks such as beer, Japanese *sake*, low-class distilled spirit, wine, whisky and brandy; seasonings such as soy sauce, *miso* and sweet *sake*; and fuel alcohols. As the method of the invention for producing brewed products, brewing processes relating to the above-mentioned brewed products are included.

Hereinbelow, the present invention will be described in more detail with reference to the following Examples, which should not be construed as limiting the scope of the invention.

[Example 1] Cloning of the *FLO1* Homologous Gene Deeply Involved in Brewer's Yeast-Type Flocculation

(1) Search for Genes Involved in the Flocculating Property of Brewer' s Yeast

For the purpose of searching for genes involved in the flocculating property of brewer's yeast, the following experiment was carried out. From the flocculent brewer's yeast strain KI084, spores were allowed to form by the method of Stewart et al. [J. Inst. Brew., 93, 216-219, (1987)] to thereby prepare strains in which chromosome number was reduced (hereinafter, such strains are called "meiotic segregants"). Of the resultant meiotic segregants, 6 strains were cultured in the medium shown in Table 1 at 20 °C under static conditions for 48 hours. After cultivation, cells were harvested by centrifugation, washed with 0.1 M EDTA twice and with sterilized water twice, and then resuspended in sterilized water. Judgment of the flocculating property of cells was carried out by the following method. Briefly, cells were suspended in flocculation measurement buffer (50 mM sodium acetate, 0.1% calcium chloride, pH 4.6) to give a final OD of OD600=2.0., left at room temperature for 30 minutes and then agitated vigorously for 20 seconds. Cells were left stationary for another 5 minutes, and then flocculation or non-flocculation was judged with the eye. As a result, the 6 meiotic segregant strains tested were grouped into 2 non-flocculent strains and 4 flocculent strains.

Medium for Flocculation Measurement	
Galactose	50 g/liter
YNB w/o AA & AS *	1.7 g/liter
Amino acids	
Aspartic acid	100 mg/ml
Glutamic acid	100 mg/ml
Threonine	100 mg/ml
Serine	100 mg/ml
Lysine hydrochloride	100 mg/ml
Arginine	100 mg/ml

* Yeast nitrogen base not containing amino acids nor ammonium sulfate. In the culturing of transformants, the above medium supplemented with adenine sulfate, tryptophan and histidine hydrochloride each at a rate of 20 mg/ml was used.

These strains were subjected to Southern analysis and Northern analysis as described blow. The extraction of total DNA was performed by culturing cells under shaking in YPD medium [2% bacto-peptone (Difco), 1% yeast extract (Difco), 2% glucose] at 30°C and extracting the total DNA from those cells which reached stationary phase according to the method of Hereford et al [Cell, 18, 1261-1271, (1979)]. Two micrograms of the extracted DNA was digested with HindIII (Boehringer Mannheim) and subjected to electrophoresis using 1% agarose gel. Then, the DNA was blotted onto a nylon filter, Hybond N+ (Amersham), according to the protocol attached thereto and then subjected to the subsequent Southern analysis. On the other hand, the extraction of total RNA was performed on these strains by culturing them under static conditions in the medium shown in Table 1 at 20 °C for 48 hours and extracting the total RNA according to the method of Villeneve and Meyer [Cell, 48, 25-37 (1987)]. Ten micrograms of the thus obtained RNA was glyoxalized by treating in 16 µl of glyoxasl/DMSO solution [1 M glyoxal, 50% DMSO, 10 mM sodium phosphate buffer (pH 7.0)] for 1 hour at 50 °C. Thereafter, 2 µl of loading buffer [50% (w/v) glycerol, 10 mM phosphate buffer (pH 7.0), 0.4% (w/v) Bromophenol Blue] and 1 µl of 1 mg/ml ethidium bromide solution were added thereto, and the resultant solution was electrophoresed in a gel containing 10 mM sodium phosphate buffer (pH 7.0) and 1% agarose. During this electrophoresis, the buffer in the electrophoresis layer was constantly circulated by using a peristaltic pump to thereby avoid generation of pH gradient. When Bromophenol Blue reached about 70% of the length of the gel, electrophoresis was terminated. Then, RNA in the gel stained with ethidium bromide was observed using a UV transilluminator and it was confirmed that the RNA had not been degraded using ribosomal RNA as an indicator. Thereafter, the RNA in the gel was blotted onto a nylon filter, Genescreen-Plus (DuPont), according to the protocol attached thereto, and this filter on which the RNA had been blotted was treated at 80 °C for 2 hours. Then, the filter was subjected to Northern analysis according to the protocol attached to Genscreen-Plus.

Partial length DNA fragments of the *FLO1* gene which were used in Southern and Northern analyses as probes were prepared as follows. Based on the nucleotide sequence for the *FLO1* gene reported by Teunissen et al. [Yeast, 9, 423-427 (1993)], two primers of 5'GATGAAACTGTCATTGTTGTCAAA3' and 5'TCGTTTCAGCAGCTAAAGTAT3' were synthesized. With these primers, PCR was carried out using the total DNA of the flocculent strain ABXL-1D (a, *FLO1*, Yeast Genetic Stock Center) as a template, and the total PCR products were electrophoresed in 1% agarose gel. An amplified DNA fragment of 1045 bp (hereinafter referred to as the "*FLO1* partial length fragment") was cut out from the gel and this fragment was recovered by using Prep-A-Gene (Bio-Rad). This fragment was labelled with [α-^{32}P] dCTP (Amersham) and used as a probe. The detection of radioactivity was carried out using an X-ray film.

The results are shown in Fig. 1. As a result of southern analysis, there were detected in the parent strain KI084 four HindIII fragments of about 9.5 kb, 5.4 kb, 4.8 kb and 3.7 kb having homology to the *FLO1* gene. Of these fragments,

two fragments of about 4.8 kb and 3.7 kb were detected in all of the KI084-derived meiotic segregants tested. Further, only in four meiotic segregants which were judged flocculent in the flocculation judging test, a fragment of about 9.5 kb was also detected in addition to the common bands. Also, as a result of Northern analysis, the transcription product of the *FLO1* gene was observed only in the parent strain and the four meiotic segregants which were judged flocculent in the flocculation judging test. From these results, it has been suggested that only the *FLO1*-homologous gene a part or the full length of which is contained in the HindIII fragment of about 9.5 kb among the three HindIII fragments homologous to the *FLO1* gene is transcribed in KI084-derived meiotic segregants, and that only those strains which have this homologous gene exhibit flocculating property. Hereinafter, the *FLO1* -homologous gene a part or the full length of which is contained in the HindIII fragment of about 9.5 kb of the KI084 strain is designated as Lg-*FLO1* (Lager Type-*FLO1*).

(2) Preparation of a Restriction Map of Lg-*FLO1*

Of KI084-derived meiotic segregants, one flocculent strain, KMS004, and one non-flocculent strain, KMS001, were selected. Their DNA's were prepared as described above and subjected to Southern analysis using several restriction enzymes independently or in combination of two enzymes and using as a probe the *FLO1* partial length fragment described above. As a result, one band which was not observed in the non-flocculent meiotic segregant was always observed in the flocculent KMS004 strain in addition to two bands that were common with the non-flocculent meiotic segregant. It was considered that a part or the full length of Lg-*FLO1* is contained in this band specific to the flocculent meiotic segregant. Thus, the length of this fragment was determined and a restriction map as shown in Fig. 2 was prepared.

(3) Cloning of a KpnI Fragment Containing a Partial Length of Lg-*FLO1*

Based on the restriction map shown in Fig. 2, the cloning of a KpnI fragment of about 5.6 kb was attempted. The DNA of the flocculent meiotic segregant KMS004 derived from KI084 was completely digested with KpnI (Boehringer Mannheim) and then fractionated by electrophoresis using 0.8% agarose. A mixture of DNA fragments of about 5.6 kb was cut out from the gel and purified by electroelution in a dialysis tube. As a result of Southern analysis using the above-mentioned *FLO1* partial length fragment as a probe, it was confirmed that the DNA fragment of interest was contained in the purified DNA fragment mixture. Then, plasmid pUC18 (Takara Shuzo) completely digested with KpnI was ligated to the purified DNA fragment mixture using DNA Ligation Kit (Takara Shuzo), and then *E. coli* DH5α (BRL) was transformed with the resultant plasmid. Of the resultant transformants obtained, 5000 strains were blotted on a nylon filter Hybond N+ (Amersham) according to the protocol attached to the filter, and colony hybridization was carried out using the above-mentioned *FLO1* partial length fragment as a probe to thereby obtain 10 positive strains. Plasmids were prepared from these strains by the alkali method and analyzed with restriction enzymes. As a result, the plasmids contained in these strains were confirmed to have the same insertion fragment. The insertion fragment of plasmid pKF-Kpn11 from one of the above strains was subjected to Southern analysis using as control the DNA's of the flocculent meiotic segregant KMS004 and the non-flocculent meiotic segregant KMS001. As a result, it could be confirmed that the insertion fragment is a part of the Lg-*FLO1* gene of interest.

(4) Partial Sequencing of the Nucleotide Sequence for a KpnI Fragment Containing a Partial Length of Lg- *FLO1*

In order to determine the nucleotide sequence for the insertion fragment from pKF-Kpn11, deletion series of the insertion fragment from pKF-Kpn11 were prepared using a deletion kit for kilosequence (Takara Shuzo) in accordance with the protocol attached to the kit. The nucleotide sequence was determined with a DNA sequencer (Perkin Elmer) using PCR/Sequencing Kit (Perkin Elmer). The nucleotide sequence was analyzed using DNASIS (Hitachi Software Engineering). The nucleotide sequence of 2.9 kb from KpnI site to HindIII site in which a coding region homologous to the coding region of known *FLO1* had been found was determined from both directions. An ORF of 2.6 kb covering from the middle of the coding region of Lg-*FLO1* to the termination codon was found in the determined nucleotide sequence.

(5) Acquisition of the Full Length of Lg-*FLO1* by Inverse-PCR

The acquisition of the full length of Lg-*FLO1* by inverse-PCR is shown typically in Fig. 3. From the previously determined nucleotide sequence for the partial length of Lg- *FLO1* [(1) in Fig. 3], primer 5 [5'AATACACAACATGGTGTCCT3', (2) in Fig. 3] and primer8 [5'ACCAGAGGTGGAACTACTGG3', (3) in Fig. 3] were synthesized. DNA from the flocculent meiotic segregant strain KMS004 (60 μg) was digested with 300 units of HindIII (Boehringer Mannheim), recovered by ethanol precipitation and dissolved in 30 μl of TE buffer. Self-ligation of the DNA fragments was carried out using DNA Ligation Kit (Takara Shuzo) in a scale of 300 μl. As a result, it is expected that cyclic molecules in which the HindIII sites represented by (4) and (5) in Fig. 3 were ligated are existing in the resultant reaction products. These reaction products

were recovered by ethanol precipitation. Using 4 μg of these reaction products as a template and the above primer5 [(2) in Fig. 3] and primer8 [(3) in Fig. 3] as primers, an inverse-PCR was carried out with LA-PCR Kit (Takara Shuzo). The composition of the reaction solution was as recommended in the protocol attached to the kit. The reaction was conducted as follows using DNA Thermal Cycler 480 (Perkin Elmer): 1 cycle at 94°C for 1 minute followed by 30 cycles at 98 °C for 20 seconds and at 68°C for 10 minutes. As a result of the electrophoresis of the reaction products using 0.8% agarose, it was observed that DNA fragments of about 8.2 kb, about 3.6 kb and about 3.0 kb were amplified.

Of these DNA fragments, the fragment of about 8.2 kb [(6) in Fig. 3] was cut out from the gel and purified using Prep-A-Gene (Bio Rad) and according to the protocol attached thereto. Since this fragment contained the BamHI, EcoRI and XbaI sites indicated in the restriction map shown in Fig. 2, it was judged that a not-yet-obtained portion of Lg-*FLO1* is contained in this fragment. This DNA fragment was digested with AluI (Boehringer Mannheim), ligated to the HincII site of pUC118 (Takara Shuzo) and introduced into *E. coli* DH5 strain (Toyobo). Plasmids were prepared from 30 strains of the resultant transformants, and the sizes of insertion fragments were examined. As a result, these plasmids could be classified into 24 groups. With respect to these plasmids, the nucleotide sequence for the insertion fragment was determined by the method as described above. As a result, one clone was obtained which has an insertion fragment of 467 bp highly homologous to the amino terminal portion of known *FLO1*. This plasmid was designated as KF1. The location of the insertion fragment of KF1 in the chromosome is represented by (7) in Fig. 3.

However, the insertion fragment of KF1 did not contain a sequence which appears to be a translation initiation site. Based on the nucleotide sequence for KF1, primerKN-2 [5'TTGTATCGGAGTATTTATA3', (8) in Fig. 3] was synthesized. Subsequently, using the template used in the above inverse-PCR and using as primers the above primer5 [(2) in Fig. 3] and primerKN-2 [(8) in Fig. 3], inverse PCR was carried out with GeneAmp PCR Reagent Kit (Takara Shuzo). The composition of the reaction solution was as recommended in the protocol attached to the kit. The reaction was conducted as follows using DNA Thermal Cycler 480: 30 cycles at 94°C for 1 minute, at 55°C for 2 minutes and at 72°C for 2 minutes followed by 1 cycle at 72°C for 10 minutes. As a result of the electrophoresis of the reaction products using 0.8% agarose, it was observed that DNA fragments of about 4.4 kb, about 1.1 kb and about 0.6 kb were amplified. Of these fragments, the DNA fragment of about 4.4 kb [(9) in Fig. 3] was cut out from the gel, purified by the method described above, blunt-ended with Klenow fragment (Takara Shuzo), ligated to the HincII site of pUC118 and introduced into *E. coli* DH5 strain. KF14, which is a plasmid of one of the transformants obtained, contained the BamHI, EcoRI and XbaI sites indicated in the restriction map shown in Fig. 2. Therefore, it was judged that the translation initiation site of Lg-*FLO1* and its 5' upstream region are contained in this fragment.

For the purpose of partially determining the nucleotide sequence for the insertion fragment of KF14 from the EcoRI site [represented by (10) in Fig. 3] toward 3', KF14 was digested with EcoRI and then a self-ligated plasmid, KF14 Δ Ec, was constructed. This plasmid has a fragment extending from the EcoRI site represented by (10) in Fig. 3 to the annealing site of primerKN-2 represented by (8) in Fig. 3. The nucleotide sequence for the insertion fragment of KF14 Δ Ec was partially determined from the EcoRI site. Based on the nucleotide sequence obtained, primerKT5'Ec [5'AGCGGTCGACCTAATAAAGGAAAAGGGGAA3', (11) in Fig. 3] was synthesized. Also, based on a part of the previously determined nucleotide sequence for the insertion fragment of pKF-Kpn11, primerKT3'Hd [5'GGAAGCTTTTTTG-TAAAACAGATTTTTTGCCCCGCTT3', (12) in Fig. 3] was synthesized. Using these two primers and using 2 μg of DNA from the flocculent meiotic segregant strain KMS004 as a template, PCR was carried out with LA-PCR Kit. The reaction was conducted as follows: 1 cycle at 94°C for 1 minutes followed by 30 cycles at 98°C for 20 seconds and at 68°C for 10 minutes. As a result of the electrophoresis of the reaction products using 0.8% agarose, it was observed that a DNA fragment of about 9 kb [(13) in Fig. 3] was amplified. Since this fragment contained the BamHI and XbaI sites indicated in the restriction map shown in Fig. 2, it was judged that the full length of Lg-*FLO1* is contained in this fragment. Hereinafter, this PCR fragment is called the Lg-*FLO1* full length fragment. The Lg-*FLO1* full length fragment was digested with several restriction enzymes and the resultant digests were electrophoresed using 0.8% agarose gel. Then, the sizes of restriction fragments were determined and a restriction map was prepared. Fig. 4 shows a restriction map of the the Lg-*FLO1* full length fragment.

(6) Introduction of the Lg-*FLO1* Full Length Fragment into Yeast and Characterization of Flocculating Property

As a host yeast strain for examination of phenotype changes due to the introduction of Lg-*FLO1*, KY644 strain was used which is an *FLO1*-disrupted strain prepared by the following procedures. Briefly, an *FLO1* partial length fragment was ligated to pRS405 (Stratagene) between the BamHI and HindIII sites. This plasmid was cut at the sole BstEII site which exists only in the insertion fragment and then introduced into a flocculent yeast strain, KY642 (a, *ura3*, *leu2*, *FLO8*), by the lithium method, to thereby obtain a strain which has become non-flocculent due to homologous recombination at the *FLO1* locus. It was confirmed by Southern analysis that the *FLO1* gene of this strain had been disrupted by the introduction of pRS405. This strain was designated as KY644 strain.

The Lg-*FLO1* full length fragment has been PCR-amplified with primers which had been so designed to give the fragment a SalI site at 5' end and a HindIII site at 3' end. This fragment was digested with SalI and HindIII. As a cloning vector, pYT37 was used which had been obtained by introducing into the EcoRI site of YIp5 the following two fragments:

a fragment containing the nucleotide sequence for CEN3 obtained from Entrez (National Center for Biotechnology Information), a databank for DNA sequences, as well as a CEN3 of 1.2 kb obtained by PCR based on the whole nucleotide sequence for the yeast chromosome No. 3, and a fragment containing an ARS sequence obtained as a YRP7-derived EcoRI-HindIII fragment. The Lg-*FLO1* full length fragment was ligated to pYT37 between the SalI and HindIII sites, and then introduced directly into KY644 strain by the lithium method.

DNA's from the resultant transformants were subjected to Southern analysis. As a result, it was confirmed that the Lg-*FLO1* full length fragment was introduced in one strain. The plasmid contained in this strain (designated as KY650) was designated as KTYT2. With respect to KY650 strain and a strain (designated as KY652 strain) obtained by introducing only the vector pYT37 into KY650, characterization of flocculating property was carried out by the method as previously described after they were cultured until reaching stationary phase in the medium shown in Table 1 at 20 °C under shaking. In order to examine inhibition of flocculation by sugars, sugars were added to the flocculation measurement buffer at the final concentration of 1 M. The results are shown in Table 2.

Table 2

Flocculating Property of Lg-*FLO1* Full Length Fragment-Introduced Yeast Strain		
Strain	KY650	KY652 (Control)
No sugar	+	-
Mannose	-	-
Glucose	-	-
Maltose	-	-
Galactose	+	-
Fructose	-	-
"+" represents flocculent and "-" non-flocculent.		

While KY652 strain did not exhibit flocculating property under any of the conditions, KY650 strain containing the Lg-*FLO1* full length fragment exhibited flocculating property in the flocculation measurement buffer when no sugar was added. This flocculating property was inhibited by mannose, glucose and maltose and somewhat inhibited by fructose, but was not inhibited by galactose. From these results, it was concluded that, by introducing the Lg-*FLO1* full length fragment, it is possible to confer on laboratory yeast the brewer's yeast-type flocculating property.

[Example 2] Acquisition of the Coding Region of Lg-*FLO1* by PCR, Introduction of the Same into Laboratory Yeast and Evaluation

With respect to an adjacent region to the part ligated to the vector in the insertion fragment of KF14, the nucleotide sequence was determined by the method described above. As a result, a site which appeared to be the translation initiation site of Lg- *FLO1* was found 49 bp upstream to 5' of the insertion fragment of KF1. PrimerKTF7 [5'CCCCAAGCTTGCTCTGCAGTAAATTCCGCA3', (14) in Fig. 3] to be used for PCR from 58 bp upstream to 5' of this initiation codon in the direction toward 3' was synthesized. Also, based on the previously determined nucleotide sequence for the insertion fragment of pKF-Kpn11, primerKTORFA [5'CGGAATTCTAAACACTATAAGCGTGATGATAG3', (15) in Fig. 3] to be used for PCR from 53 bp downstream to 3' of the termination codon of the Lg-*FLO1* coding region in the direction toward 5' was synthesized. Using these two primers and using 2 μg of DNA from the flocculent meiotic segregant strain KMS004 as a template, PCR was carried out with LA-PCR Kit. The reaction was conducted by repeating 30 cycles of at 94°C for 30 seconds, at 60°C for 1 minute and at 72°C for 3.5 minutes. As a result of electrophoresis of the reaction products using 0.8% agarose, it was observed that a DNA fragment of about 5.8 kb [(16) in Fig. 3] was amplified. Hereinafter, this PCR fragment is called the Lg-*FLO1*ORF fragment. The Lg-*FLO1*ORF fragment was PCR-amplified with primers which had been so designed to give the fragment a HindIII site at 5' end and a EcoRI site at 3' end. This fragment was digested with HindIII and EcoRI and ligated to the yeast expression vector pYES2 (Invitrogen) between the HindIII and EcoRI sites in such a manner that the fragment was inserted in the downstream of the *GAL1* promoter in the sense direction. Then, the vector was directly introduced to the KY644 strain described above by the lithium method. DNA's from the resultant transformants were subjected to Southern analysis, and one of those

strains in which the introduction of the Lg-*FLO1*ORF fragment had been confirmed was designated as KY646 strain. Also, the plasmid contained in KY646 strain was designated as YESKT2. With respect to KY646 strain and a strain (designated as KY649 strain) obtained by introducing only the vector pYES2 into KY644 strain, characterization of flocculating property was carried out by the method as previously described after they were cultured until reaching stationary phase in the medium shown in Table 1 at 20 °C under shaking. The results are shown in Table 3.

Table 3

Flocculating Property of Yeast Strain into which *GAL1* Promoter-Controlled Lg-*FLO1*ORF Fragment is Introduced		
Strain	KY646	KY649 (Control)
No sugar	+	-
Mannose	-	-
Glucose	-	-
Maltose	-	-
Galactose	+	-
Fructose	-	-
"+" represents flocculent and "-" non-flocculent.		

While KY649 strain did not exhibit flocculating property under any of the conditions, KY646 strain containing the Lg-*FLO1*ORF fragment exhibited flocculating property in the flocculation measurement buffer when no sugar was added. This flocculating property was, similar to that of KY650 strain, the brewer's yeast-type flocculating property. In other words, this flocculating property was inhibited by mannose, glucose and maltose and somewhat inhibited by fructose, but was not inhibited by galactose. From these results, it was concluded that, by introducing the Lg-*FLO1*ORF fragment controlled by *GAL1* promoter, it is possible to confer on laboratory yeast the brewer's yeast-type flocculating property. In other words, it was concluded that the coding region of the Lg-*FLO1* gene exists in the Lg-*FLO1*ORF fragment.

[Example 3] Specification of the Region in Lg-*FLO1* Controlling Brewer's Yeast-Type Flocculation

In order to specify the region in Lg-*FLO1* which controls the brewer's yeast-type flocculation, a chimeric gene composed of Lg-*FLO1* and Sc-*FLO1* [laboratory yeast-type *FLO1* disclosed by Watari et al., Yeast, 10, 211-225 (1994)] was created by the method as shown in Fig. 5 and investigated into the flocculating property thereof. The Lg-*FLO1* ORF fragment was digested with XhoI and KpnI, blunt-ended with Klenow fragment and then cloned into the HincII site of pUC118. Of the resultant transformants, one clone having an insertion fragment of about 1 kb was selected and the nucleotide sequence for the insertion fragment was determined. Based on the result, primerKTF8 (5'CGGGATCCATCTGGCAATACCACACTAACA3') was synthesized which extends from 639 bp downstream to 3' of the initiation codon of Lg-*FLO1* toward 5'. Using primerKTF7 and primerKTF8 and using as a template 2 μg of DNA from the flocculent meiotic segregant strain KMS004, PCR was carried out with LA-PCR Kit. The reaction was conducted by repeating 30 cycles of at 94°C for 30 seconds, at 60°C for 1 minute and at 72°C for 3.5 minutes. As a result of electrophoresis of the reaction products using 0.8% agarose, it was observed that a fragment of about 0.7 kb [(16) in Fig. 3] was amplified. Hereinafter, this PCR fragment is called the Lg-*FLO1* N-terminal fragment. The fragment obtained was cloned into pT7Blue (Novagen) which is a cloning vector for PCR products. Using four independent clones obtained, the nucleotide sequences for their insertion fragments were determined from both directions. All of these four clones were found to have the same insertion fragment. The nucleotide sequence obtained is shown in SEQ ID NO: 3. One of these clones was designated as KNTA1. The Lg-*FLO1* N-terminal fragment was PCR-amplified with primers which had been so designed to give the fragment a HindIII site at 5' end and a BamHI site at 3' end. KNTA1 was digested with HindIII and BamHI and subjected to electrophoresis. Then, the insertion fragment separated from the vector was cut out from the gel, purified by the method as described above and cloned into the HindIII-BamHI site of pYES2 in such a manner that the fragment was inserted in the downstream of the *GAL1* promoter in the sense direction. The plasmid obtained was designated as KNYES. *Escherichia coli* EKB707 containing this plasmid KNYES was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology

(1-3, Higashi 1-Chome, Tsukuba City, Ibaragi Pref., Japan) on January 27, 1995 under the accession No. FERM BP-4983.

Based on the nucleotide sequence for the laboratory yeast-type *FLO1* gene (hereinafter referred to as the "Sc-*FLO1* gene") which Watari et al. disclosed [Yeast, 10, 211-225 (1994)], primerWtF1N (5'CGGGATCCACTGTAAGTGATGACTTCGAAG3') extending from 721 bp downstream to 3' of the initiation codon toward 3' and primerFLID4 (5'CGGAATTCTCAGCGTATAATTAGCAAAGAA3') extending from 58 bp downstream to 3' of the termination codon toward 5' were synthesized. Using these primers and using as a template 2 μg of DNA from ABXL-1D strain, PCR was carried out with LA-PCR Kit. The reaction was conducted by repeating 30 cycles of at 94°C for 30 seconds, at 60°C for 1 minute and at 72°C for 3.5 minutes. As a result of electrophoresis of the reaction products using 0.8% agarose, it was observed that a fragment of about 3.9 kb was amplified. Hereinafter, this fragment is called the Sc-*FLO1* C-terminal fragment. The Sc-*FLO1* C-terminal fragment was PCR-amplified with primers which had been so designed to give the fragment a BamHI site at 5' end and an EcoRI site at 3' end. This fragment was digested with BamHI and EcoRI, and ligated to the previously constructed KNYES between BamHI and EcoRI sites in such a manner that the fragment was inserted in the downstream of the Lg-*FLO1* N-terminal fragment in the sense direction. As a result, it is expected that a gene coding for a chimeric *FLO1* protein is constructed in which a portion of the Sc-*FLO1* coding region corresponding to the amino acid sequence from the amino terminal to position 339 has been replaced with a portion of the Lg- *FLO1* gene corresponding to the amino acid sequence from the amino terminal to position 312. The ligation reaction product was introduced directly to the KY644 strain described above by the lithium method. DNA's from the resultant transformants were subjected to Southern analysis. Of those strains in which the introduction of the chimeric gene composed of the Lg-*FLO1* N-terminal fragment and the Sc-*FLO1* C-terminal fragment was confirmed, one strain was designated as KY648 strain. Also, the plasmid contained in this strain was designated as KNWtC3.

Further, primerFLID1 (5'CCCCAAGCTTTCGTTTGATGTAAGCTCTCT3') which is extending from -69 bp upstream to 5' of the initiation codon of Sc-*FLO1* toward 3' was synthesized. Using primerFLID1 and primerFLID4 and using as a template 2 μg of DNA from ABXL-1D strain, PCR was carried out with LA-PCR Kit. The reaction was conducted by repeating 30 cycles of at 94°C for 30 seconds, at 60°C for 1 minute and at 72°C for 3.5 minutes. As a result of electrophoresis of the reaction products using 0.8% agarose, it was observed that a fragment of about 4.8 kb was amplified. Hereinafter, this fragment is called the Sc-*FLO1*ORF fragment. The Sc-*FLO1*ORF fragment was PCR-amplified with primers which had been so designed to give the fragment a HindIII site at 5' end and an EcoRI site at 3' end. This fragment was digested with HindIII and EcoRI, and ligated to pYES2 between the HindIII and EcoRI sites in such a manner that the fragment was inserted in the downstream of *GAL1* promoter in the sense direction. The resultant vector was introduced directly to the above-mentioned KY644 strain by the lithium method. DNA's from the resultant transformants were subjected to Southern analysis. Of those strains in which the introduction of the Sc-*FLO1*ORF fragment was confirmed, one strain was designated as KY647 strain and used for the comparison of flocculating properties. Also, the plasmid contained in this strain was designated as YESWt1.

With respect to KY647 strain, KY648 strain and the previously described KY649 strain, characterization of flocculating property was carried out by the method as previously described after they were cultured until reaching stationary phase in the medium shown in Table 1 at 20 °C under shaking. The results are shown in Table 4.

Table 4

Flocculating Property of Yeast Strain into which *GAL1* Promoter-Controlled Lg-Sc Chimeric *FLO1* Gene is Introduced			
Strain	KY648	KY649 (Control)	KY647 (Comparison)
No sugar	+	-	+
Mannose	-	-	-
Glucose	-	-	+
Maltose	-	-	+
Galactose	+	-	+
Fructose	-	-	+
"+" represents flocculent and "-" non-flocculent.			

While KY649 strain did not exhibit flocculating property under any of the conditions, KY648 strain containing the chimeric gene composed of the Lg-*FLO1* N-terminal fragment and the Sc-*FLO1* C-terminal fragment and KY647 strain

containing the Sc-*FLO1*ORF fragment exhibited flocculating property in the flocculation measurement buffer when no sugar was added. The flocculating property of KY648 was, similar to that of KY650, inhibited by mannose, glucose and maltose and somewhat inhibited by fructose, but was not inhibited by galactose. On the other hand, the flocculating property of KY647 was only inhibited by mannose, but was not inhibited by glucose, maltose, fructose nor galactose. In other words, while the Sc-*FLO1*ORF fragment confers laboratory yeast-type flocculating property, the flocculating property conferred by the chimeric gene prepared by replacing a portion of this fragment extending from its initiation codon to 720 bp toward 3' with a portion of Lg-*FLO1* extending from the initiation codon to 639 bp toward 3' was converted to the brewer's yeast-type. From these results, it was concluded that what is deeply involved in the conferring of brewer's yeast-type flocculating property is the Lg-*FLO1* N-terminal fragment in the Lg-*FLO1*ORF fragment, i.e., the sequence shown in SEQ ID NO: 3 of the Sequence Listing.

[Example 4] Evaluation of Lg-*FLO1*-Disrupted Brewer's Yeast

(1) Preparation of a Plasmid for Disrupting Lg-*FLO1*

A Plasmid for disrupting Lg-*FLO1* was prepared as shown in Figs. 6 and 7. Briefly, plasmid pUC18 was digested with KpnI and then DNA fragments blunt-ended with Klenow fragment were self-ligated, to thereby prepare plasmid pUC18 Δ K. This plasmid was digested with HincII and then a KpnI linker (GGGTACCC) was inserted thereinto, to thereby prepare plasmid pUC18±K. Between the EcoRI and BamHI sites of this plasmid, a 0.9 kb EcoRI-BamHI fragment (containing 5' flanking region) obtained from plasmid KF14 was inserted to thereby prepare plasmid pKF5B1.

From plasmid pKF3HP which was obtained by inserting a 1.7 kb HincII-PvuII fragment (containing 3' flanking region) into the SmaI site of plasmid pUC118, a 1.7 kb BamHI-KpnI fragment was obtained and inserted between the BamHI and KpnI sites of plasmid pKF5B1, to thereby prepare plasmid pKF53-1.

Plasmid pSY114P (Japanese Unexamined Patent Publication No. 2-265488) which contains a promoter region (1.0 kb) from yeast GPD (glyceraldehyde-3-phosphate dehydrogenase) gene and a terminator region (0.4 kb) from yeast PGK (phosphoglycerate kinase) gene was digested with SmaI and then a HindIII linker (CAAGCTTG) was ligated thereto. Subsequently, the plasmid was digested with HindIII and then self-ligated, to thereby prepare plasmid pSY114H. To the HindIII site of this plasmid, a 0.5 kb HindIII fragment of pSV2bsr (Funakoshi) containing a Blasticidin S resistant gene was inserted to thereby prepare plasmid pGPDBSR. This plasmid was digested with SalI and then blunt-ended with Klenow fragment, to thereby obtain a 1.9 kb DNA fragment. This DNA fragment was ligated to a DNA fragment obtained by digesting plasmid pKF53-1 with BamHI and then blunt-ending with Klenow fragment, to thereby prepare plasmid pKF53BSR19.

(2) Transformation of Brewer's Yeast

Transformation of brewer's yeast was performed by electroporation. Flocculent brewer's yeast cultured in 200 ml of YPD medium until OD600 reached approximately 7 was washed twice with sterilized water and twice with 1 M sorbitol, and then resuspended in 1 ml of 1 M sorbitol. To 50 μl of this yeast suspension, 2.7 μg of DNA fragments obtained by digesting plasmid pKF53BSR with EcoRI and 10 μg of salmon sperm (DNA) (Sigma) were added and left for 5 minutes. Then, using a 0.2 cm cell of the Gene Pulser (Bio Rad), an electric pulse of 1.5 KV, 25 μF and 200 Ω was charged. To the resultant suspension, 1 ml of 1 M sorbitol and 400 μl of YPD were added and cultured at 30°C for 4 hours under shaking. Thereafter, the suspension was plated on YPD agar medium containing 50 μg/ml Blasticidin S (Funakoshi) and cultured at 30 °C for 3 days. The resultant transformants were subjected to Southern analysis and, as a result, it was confirmed that the Lg-*FLO1* gene was disrupted. The thus obtained Lg-*FLO1*-disrupted brewer's yeast was evaluated for flocculating property and was found to have been converted to non-flocculent.

[Example 5] Comparison of the Deduced Amino Acid Sequences of the N-Terminal Region for Lg-*FLO1* and Sc-*FLO1*

Example 3 has shown that brewer's yeast-type flocculating property is controlled by the sequence of 213 amino acid residues in the N-terminal region of Lg-*FLO1*. Then, the deduced amino acid sequences of this region for Lg-*FLO1* and Sc-*FLO1* were compared. As a result, as shown in Fig. 8, the following characteristic differences were observed between the two sequences. 1) In Lg-*FLO1*, 27 amino acid residues corresponding to those from position 84 to position 110 of Sc-*FLO1* are deleted. 2) Up to the position 123 based on Sc-*FLO1*, homology between Sc-*FLO1* and Lg-*FLO1* is relatively low. 3) At the position 124 and thereafter based on Sc-*FLO1*, homology between Sc-*FLO1* and Lg-*FLO1* is high.

Based on these results, there were prepared a chimeric gene composed of Sc-*FLO1* and Lg-*FLO1* and a modified Sc-*FLO1* gene having a deletion of the 27 amino acid residues from position 84 to position 110. The N-terminal regions of these modified *FLO1* genes were prepared using the recombinant PCR method described on pages 155-160 of PCR Experiment Manual [M.A. Innis et al. (eds.), edited and translated by Takashi Saito, HBJ Shuppan Co., Ltd. (1991)]. A

fragment of the thus prepared T-terminal region of the modified *FLO1* gene was ligated to plasmid KNWtC3 (described in Example 3) between the HindIII and BamHI sites (i.e., between *GAL1* promoter and the Sc-*FLO1* C-terminal fragment) in the sense direction, and then introduced into the yeast KY644 strain. Culture of the resultant transformants and evaluation of flocculating property were carried out in substantially the same manner as in Example 3. The results are shown in Fig. 9. Those strains (KY707, KY708 and KY709) having a chimeric *FLO1* gene composed of an Lg-*FLO1*-derived portion up to amino acid residue at position 46, 68 or 83 (based on Sc- *FLO1*) and an Sc-*FLO1*-derived portion following the above portion exhibited, similar to a strain having Sc-*FLO1* (KY706), strong laboratory yeast-type flocculation. On the other hand, a strain having a chimeric *FLO1* gene composed of an Lg-*FLO1*-derived portion up to amino acid residue at position 124 based on Sc-*FLO1* (position 97 based on Lg-*FLO1*) and an Sc-*FLO1*-derived portion following the above portion exhibited, similar to KY648 and KY646 shown in Example 3, weak brewer's yeast-type flocculation. Further, KY711 having a modified *FLO1* gene having a deletion of the 27 amino acid residues from position 84 to position 110 of Sc-*FLO1* exhibited weak laboratory yeast-type flocculation. From these results, it has been shown that the portion of the Lg-*FLO1* gene involved in brewer's yeast-type is a portion corresponding to the 14 amino acid residues from position 84 to position 97 based on Lg-*FLO1*, i.e., the sequence shown in SEQ ID NO: 4 coding for the amino acid sequence shown in SEQ ID NO: 2 in the Sequence Listing.

Industrial Applicability

According to the present invention, there are provided the Lg-Flo1 protein having an activity to confer on yeast brewer's yeast-type flocculating property as well as the Lg-*FLO1* DNA strand coding for the protein. By introducing the DNA of the invention into yeast as a foreign DNA, i.e., introducing this DNA into yeast cells as an extranuclear gene and(/or) a nuclear gene, it is possible to confer on yeast brewer's yeast-type flocculating property or enhance the property in yeast. On the contrary, by introducing a DNA obtained by disrupting the DNA of the invention into yeast cells, or by inhibiting the expression of the DNA of the invention, it is possible to convert a flocculent yeast strain into a non-flocculent yeast strain, or to reduce flocculating property.

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO: 1

SEQUENCE CHARACTERISTICS:

LENGTH: 213 amino acids

TYPE: amino acids

TOPOLOGY: linear

MOLECULAR TYPE: peptide

SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Thr Ile Ala His His Cys Ile Phe Leu Val Ile Leu Ala Phe Leu
1               5                  10                  15

Glu Leu Leu Asn Val Ala Ser Gly Ser Thr Gln Ala Cys Leu Pro Val
            20                  25                  30

Gly Ser Arg Lys Asn Gly Met Asn Val Asn Phe Tyr Lys Tyr Ser Leu
        35                  40                  45

Gln Asp Ser Thr Thr Tyr Ser Asp Pro Gln Tyr Met Ala Tyr Lys Tyr
    50                  55                  60

Ser Asp Thr Lys Lys Leu Gly Ser Val Ser Gly Gln Thr His Leu Ser
65                  70                  75                  80

Ile Tyr Tyr Gly Pro Asn Thr Ala Phe Trp Asn Thr Ala Ser Trp Ser
                85                  90                  95

Ser Asp Leu Phe Gly Phe Tyr Thr Thr Pro Thr Asn Val Thr Val
```

Glu

100 105 110

Met Thr Gly Tyr Phe Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys

115 120 125

Phe Ala Thr Val Asp Asp Ser Ala Ile Leu Ser Val Gly Gly Ser Ile

130 135 140

Ala Phe Glu Cys Cys Ala Gln Glu Gln Pro Pro Ile Thr Ser Thr Asp

145 150 155 160

Phe Thr Ile Asn Gly Ile Lys Pro Trp Asp Ala Ala Ala Pro Thr Asp

165 170 175

Ile Lys Gly Ser Thr Tyr Met Tyr Ala Gly Tyr Tyr Tyr Pro Ile Lys

180 185 190

Ile Val Tyr Ser Asn Ala Lys Val Leu Ala Arg Leu Pro Val Ser Val

195 200 205

Val Leu Pro Asp Gly

210

INFORMATION FOR SEQ ID NO: 2

SEQUENCE CHARACTERISTICS:

LENGTH: 14 amino acids

TYPE: amino acids

TOPOLOGY: linear

MOLECULAR TYPE: peptide

SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Pro Asn Thr Ala Phe Trp Asn Thr Ala Ser Trp Ser Ser
1 5 10

INFORMATION FOR SEQ ID NO: 3

SEQUENCE CHARACTERISTICS:

LENGTH: 697 base pairs

TYPE: nucleic acids

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULAR TYPE: genomic DNA

ORIGINAL SOURCE:

SPECIES: *Saccharomyces cerevisiae*

STRAIN: KMS004

FEATURE:

NAME/KEY: CDS

LOCATION: 59. . 697

IDENTIFICATION METHOD: E

SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCTCTGCAGT AAATTCCGCA AATGATTTTC TTTAAATTGA TTAGCACCAC TAAAAAAA 58

ATG ACA ATT GCA CAC CAC TGC ATA TTT TTG GTA ATC TTG GCC TTT CTG 106
Met Thr Ile Ala His His Cys Ile Phe Leu Val Ile Leu Ala Phe Leu
1 5 10 15

GAG CTA CTT AAC GTA GCA TCA GGA AGT ACA CAA GCA TGC CTG CCA GTG 154
Glu Leu Leu Asn Val Ala Ser Gly Ser Thr Gln Ala Cys Leu Pro Val
20 25 30

```
GGC TCG AGG AAA AAT GGG ATG AAT GTC AAC TTT TAT AAA TAC TCA TTA  202
Gly Ser Arg Lys Asn Gly Met Asn Val Asn Phe Tyr Lys Tyr Ser Leu
          35                  40                  45

CAG GAT TCA ACA ACG TAT TCC GAC CCG CAA TAT ATG GCC TAT AAA TAC  250
Gln Asp Ser Thr Thr Tyr Ser Asp Pro Gln Tyr Met Ala Tyr Lys Tyr
     50                  55                  60

TCC GAT ACA AAG AAG TTA GGT TCC GTT AGC GGA CAG ACC CAT CTC TCC  298
Ser Asp Thr Lys Lys Leu Gly Ser Val Ser Gly Gln Thr His Leu Ser
65                  70                  75                  80

ATA TAC TAT GGC CCA AAT ACT GCC TTT TGG AAT ACT GCC TCT TGG AGT  346
Ile Tyr Tyr Gly Pro Asn Thr Ala Phe Trp Asn Thr Ala Ser Trp Ser
                 85                  90                  95

TCT GAT CTT TTT GGT TTC TAT ACT ACT CCA ACT AAT GTA ACT GTG GAA  394
Ser Asp Leu Phe Gly Phe Tyr Thr Thr Pro Thr Asn Val Thr Val Glu
            100                 105                 110

ATG ACA GGG TAC TTT TTA CCA CCA CAG ACG GGT TCT TAC ACA TTC AAG  442
Met Thr Gly Tyr Phe Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys
        115                 120                 125

TTT GCT ACA GTT GAC GAC TCT GCA ATT TTA TCG GTT GGT GGT AGC ATT  490
Phe Ala Thr Val Asp Asp Ser Ala Ile Leu Ser Val Gly Gly Ser Ile
    130                 135                 140

GCG TTC GAA TGT TGT GCA CAA GAA CAA CCT CCT ATC ACA TCA ACG GAT  538
Ala Phe Glu Cys Cys Ala Gln Glu Gln Pro Pro Ile Thr Ser Thr Asp
145                 150                 155                 160

TTC ACT ATT AAC GGT ATT AAA CCA TGG GAC GCA GCT GCA CCT ACC GAC  586
Phe Thr Ile Asn Gly Ile Lys Pro Trp Asp Ala Ala Ala Pro Thr Asp
                165                 170                 175

ATA AAG GGG TCA ACG TAC ATG TAC GCC GGT TAC TAT TAC CCG ATC AAA  634
Ile Lys Gly Ser Thr Tyr Met Tyr Ala Gly Tyr Tyr Tyr Pro Ile Lys
```

```
            180                 185                 190
ATT GTT TAT TCA AAT GCT AAA GTC TTG GCT AGG CTT CCT GTT AGT GTG  682
Ile Val Tyr Ser Asn Ala Lys Val Leu Ala Arg Leu Pro Val Ser Val
        195                 200                 205
GTA TTG CCA GAT GGA  697
Val Leu Pro Asp Gly
    210
```

INFORMATION FOR SEQ ID NO: 4

SEQUENCE CHARACTERISTICS:

LENGTH: 42 base pairs

TYPE: nucleic acids

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULAR TYPE: genomic DNA

ORIGINAL SOURCE:

SPECIES: *Saccharomyces cerevisiae*

STRAIN: KMS004

FEATURE:

NAME/KEY: CDS

LOCATION: 1. . 42

IDENTIFICATION METHOD: E

SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
GGC CCA AAT ACT GCC TTT TGG AAT ACT GCC TCT TGG AGT TCT 42
Gly Pro Asn Thr Ala Phe Trp Asn Thr Ala Ser Trp Ser Ser
1               5                   10
```

Claims

1. A protein which comprises a polypeptide having the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing and which has an activity of conferring on yeast brewer's yeast-type flocculating property.

2. A protein which comprises a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing the amino acid residues from position 25 to position 213 and which has an activity of conferring on yeast brewer's yeast-type flocculating property.

3. A protein which comprises a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing at least the amino acid residues from position 25 to position 97 and which has an activity of conferring on yeast brewer's yeast-type flocculating property.

4. A polypeptide which has an activity of conferring on yeast brewer's yeast-type flocculating property and which has the amino acid sequence shown substantially in SEQ ID NO: 2 in the Sequence Listing.

5. A DNA comprising a nucleotide sequence coding for a polypeptide having the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing.

6. A DNA comprising a nucleotide sequence coding for a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing the amino acid residues from position 25 to position 213.

7. A DNA comprising a nucleotide sequence coding for a polypeptide having a partial sequence of the amino acid sequence shown substantially in SEQ ID NO: 1 in the Sequence Listing containing at least the amino acid residues from position 25 to position 97.

8. A DNA which comprises a nucleotide sequence coding for a protein having an activity of conferring on yeast brewer's yeast-type flocculating property and which comprises a partial sequence of the nucleotide sequence shown in SEQ ID NO: 3 in the Sequence Listing containing the bases from position 59 to 697 or a cDNA thereto.

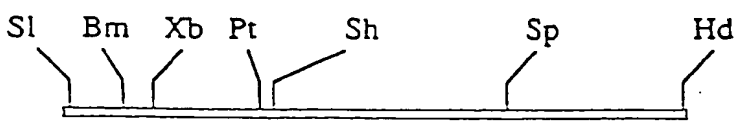
9. A DNA which comprises a nucleotide sequence coding for a protein having an activity of conferring on yeast brewer's yeast-type flocculating property and which comprises a partial sequence of the nucleotide sequence shown in SEQ ID NO: 3 in the Sequence Listing containing the bases from position 131 to position 697 or a DNA complementary thereto.

10. A DNA comprising a partial sequence of the nucleotide sequence shown in SEQ ID NO: 3 in the Sequence Listing containing the bases from position 131 to position 349 or a DNA complementary thereto.

11. A DNA which comprises the nucleotide sequence shown in SEQ ID NO: 4 in the Sequence Listing and which codes for a polypeptide having an activity of conferring on yeast brewer's yeast-type flocculating property or a cDNA thereto.

12. A DNA which is incorporated into plasmid KTYT2, YESKT2 or KNWtC3 and which comprises a nucleotide sequence coding for a protein having an activity of conferring on yeast brewer's yeast-type flocculating property.

13. The DNA according to claim 12, which is incorporated into plasmid KTYT2 and is a DNA fragment of about 9 kb having the following restriction map:



wherein Bm = BamHI, Pt = PstI, Sl = SalI, Sp = SpeI, Sh = SphI and Xb = XbaI.

14. A DNA which is incorporated into plasmid KNYES and which comprises a nucleotide sequence coding for a protein

having an activity of conferring on yeast brewer's yeast-type flocculating property.

15. A plasmid comprising the DNA according to any one of claims 5 through 14.

16. A method for producing a yeast strain wherein brewer's yeast-type flocculating property has been conferred or enhanced, characterized by introducing thereinto the DNA according to any one of claims 5 through 14.

17. A method for producing a yeast strain wherein brewer's yeast-type flocculating property has been eliminated or reduced, characterized by introducing thereinto a DNA of which the ability to express a protein having an activity of conferring brewer's yeast-type flocculating property has been eliminated or reduced by disrupting the DNA according to claim 5 or 14.

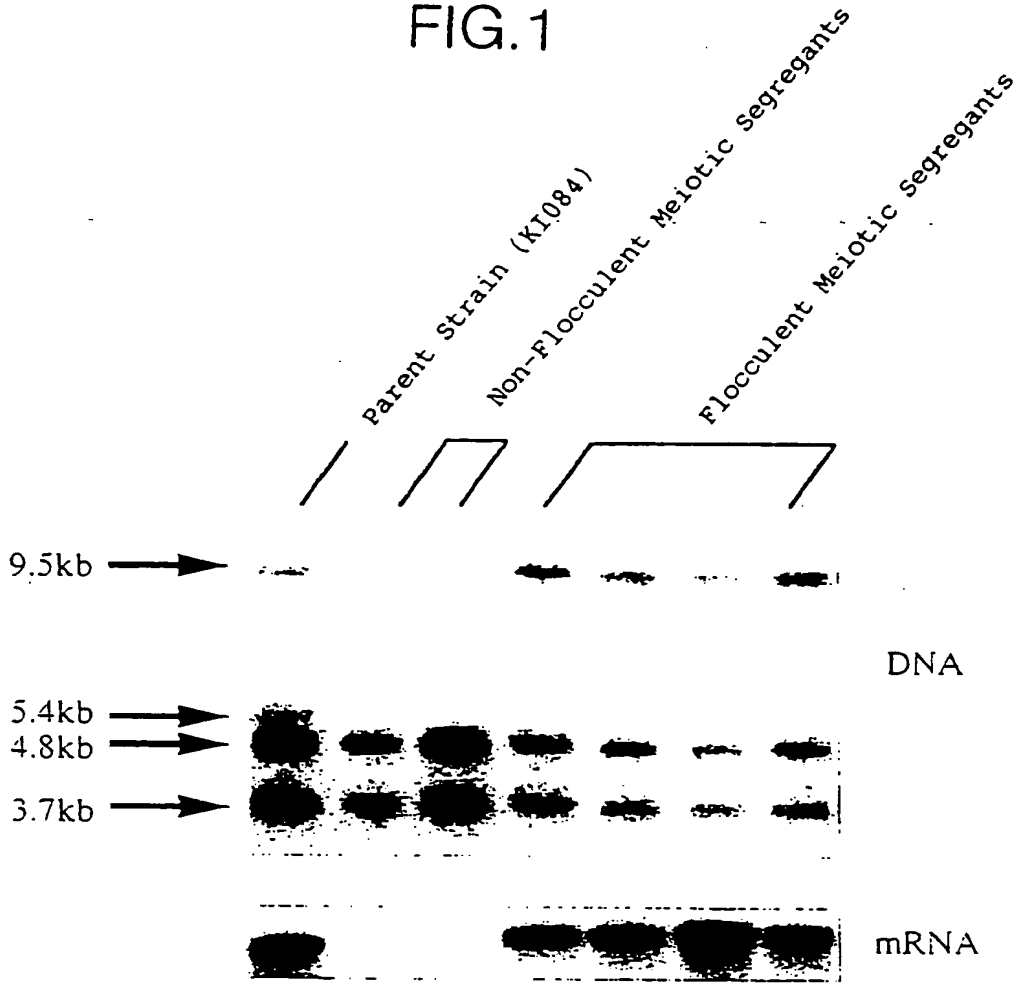
18. A method for eliminating or reducing brewer's yeast-type flocculating property in yeast by inhibiting the expression of the DNA according to any one of claims 5 through 14.

19. A yeast strain which is produced by the method according to claim 16 or 17.

20. A method for producing a brewed product comprising culturing the yeast strain according to claim 19.

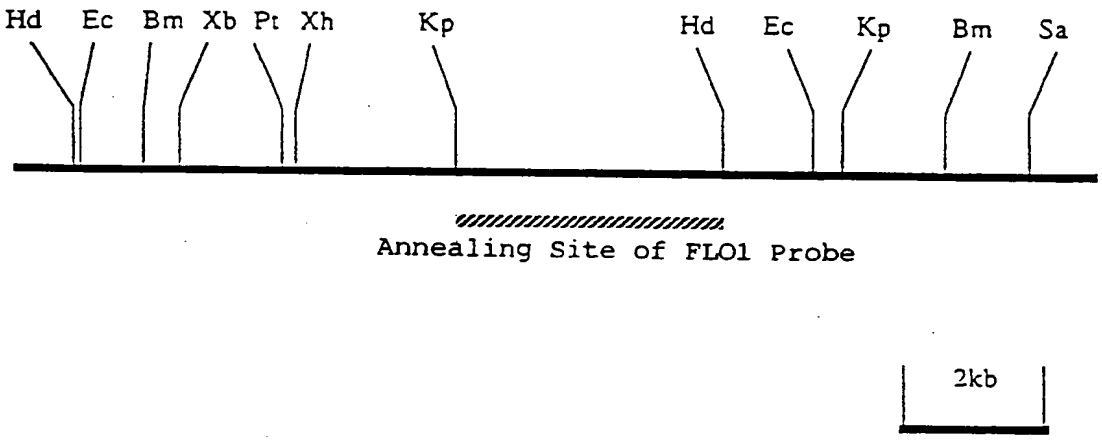
21. The brewed product produced by the method according to claim 20.

FIG.1



Results of Southern and Northern Analyses on *FLO1* Gene in Brewer's Yeast Strain and Meiotic Segregants Thereof

FIG.2

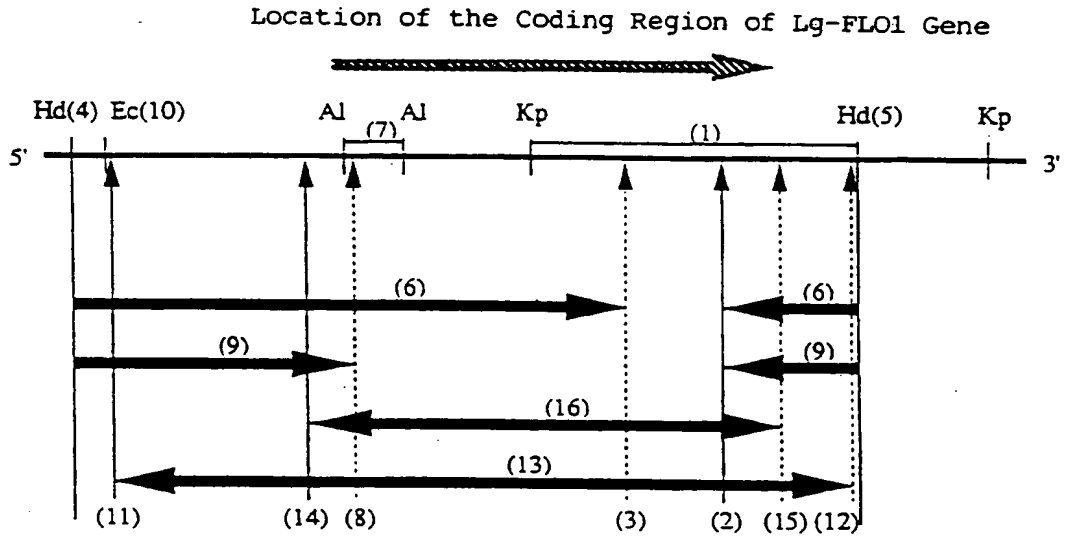


Restriction Map of Lg-*FLO1* Gene

Abbreviations represent the following restriction sites: Bm: BamHI, Ec:EcoRI, Hd:HindIII, Kp:KpnI, Pt:PstI, Sa:SalI, Xb:XbaI, Xh:XhoI.

Due to the nature of the experiment, only those restriction sites located closest to the annealing site of FLO1 probe are shown. Other sites, even if they exist, are not shown.

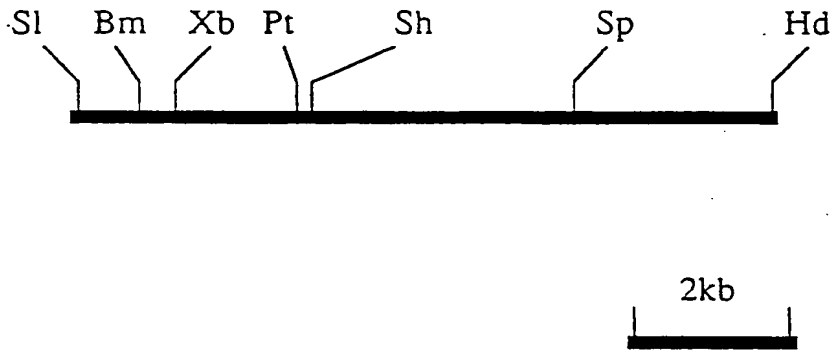
FIG.3



Concept Diagram of Cloning of the Full Length of Lg-*FLO1* by Inverse PCR

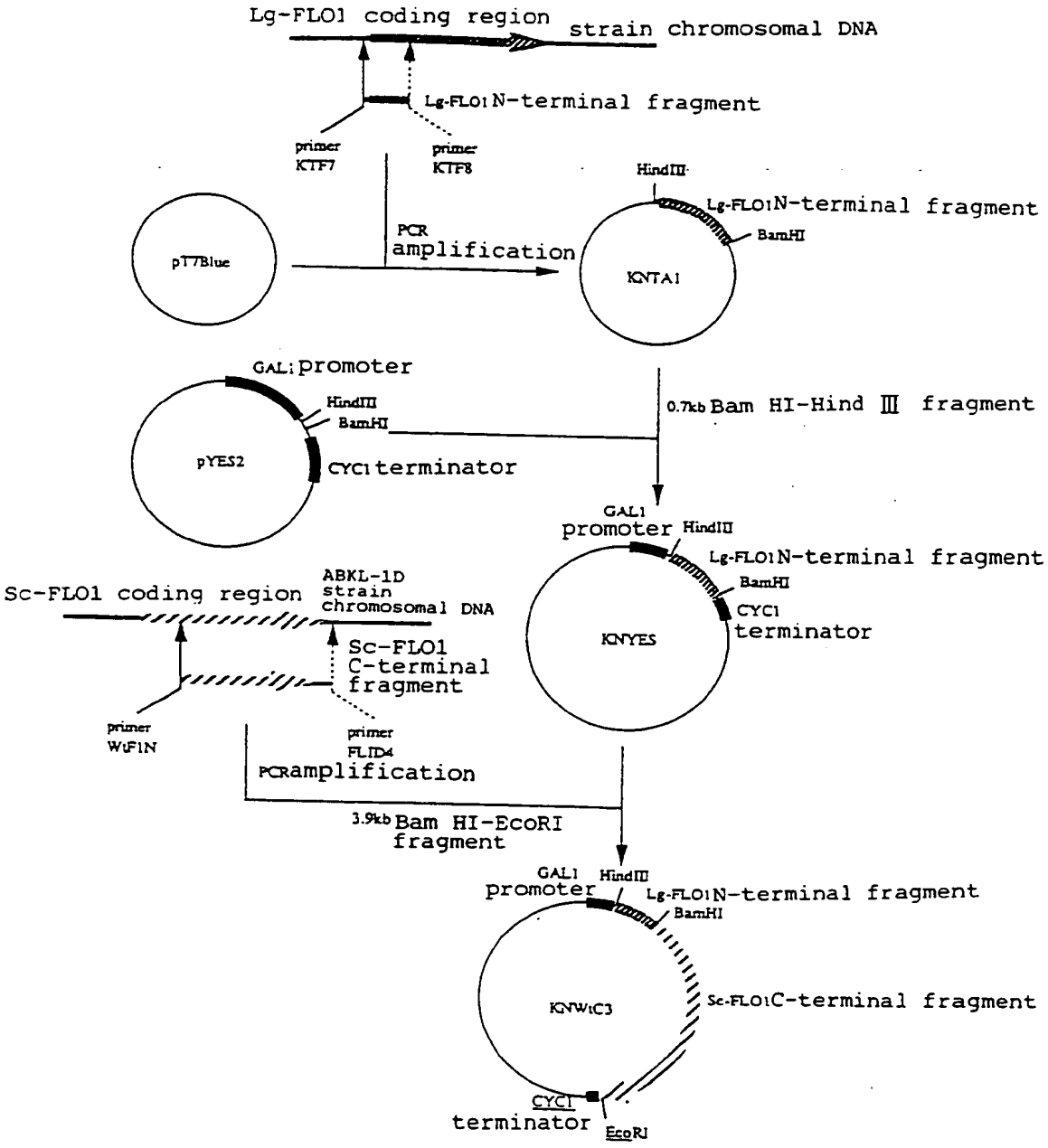
For figures in parentheses, see explanation given in the specification. Upward arrows indicate positions of primers. (Solid lines represent 5'→3' primers and dotted lines 3'→5' primers.) Bold, solid arrows represent PCR fragments. Abbreviations represent the following restriction enzymes: Hd: HindIII, Ec:EcoRI, Al:AluI and Kp:KpnI.

FIG.4



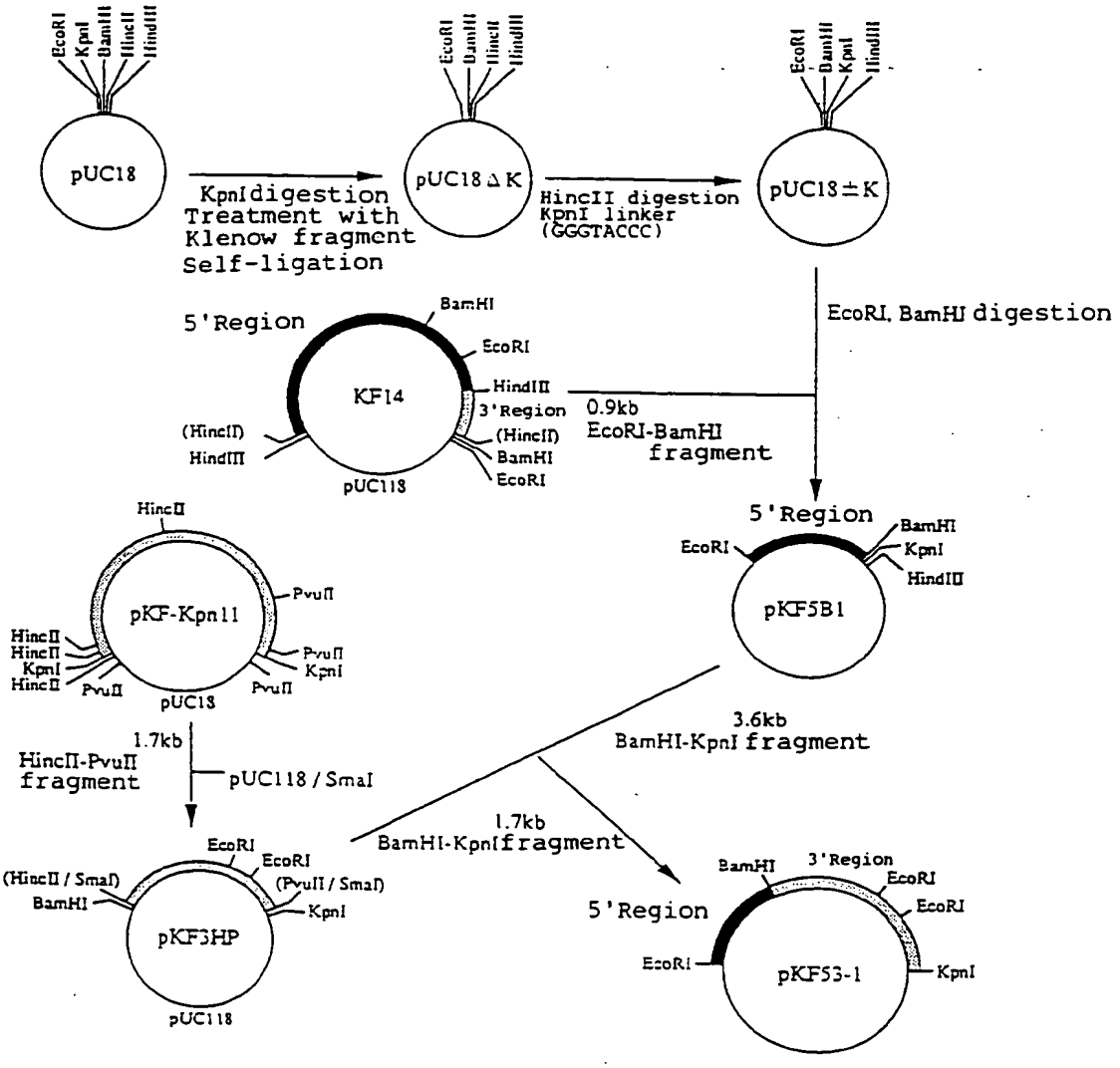
Restriction Map of Lg-*FLO1* Full Length Fragment
Abbreviations represent the following restriction sites: Bm: BamHI, Pt:PstI, Sl:SalI, Sp:SpeI, Sh:SphI, Xb:XbaI.
The SalI site has been given by primers and is not present in the chromosome.

FIG.5



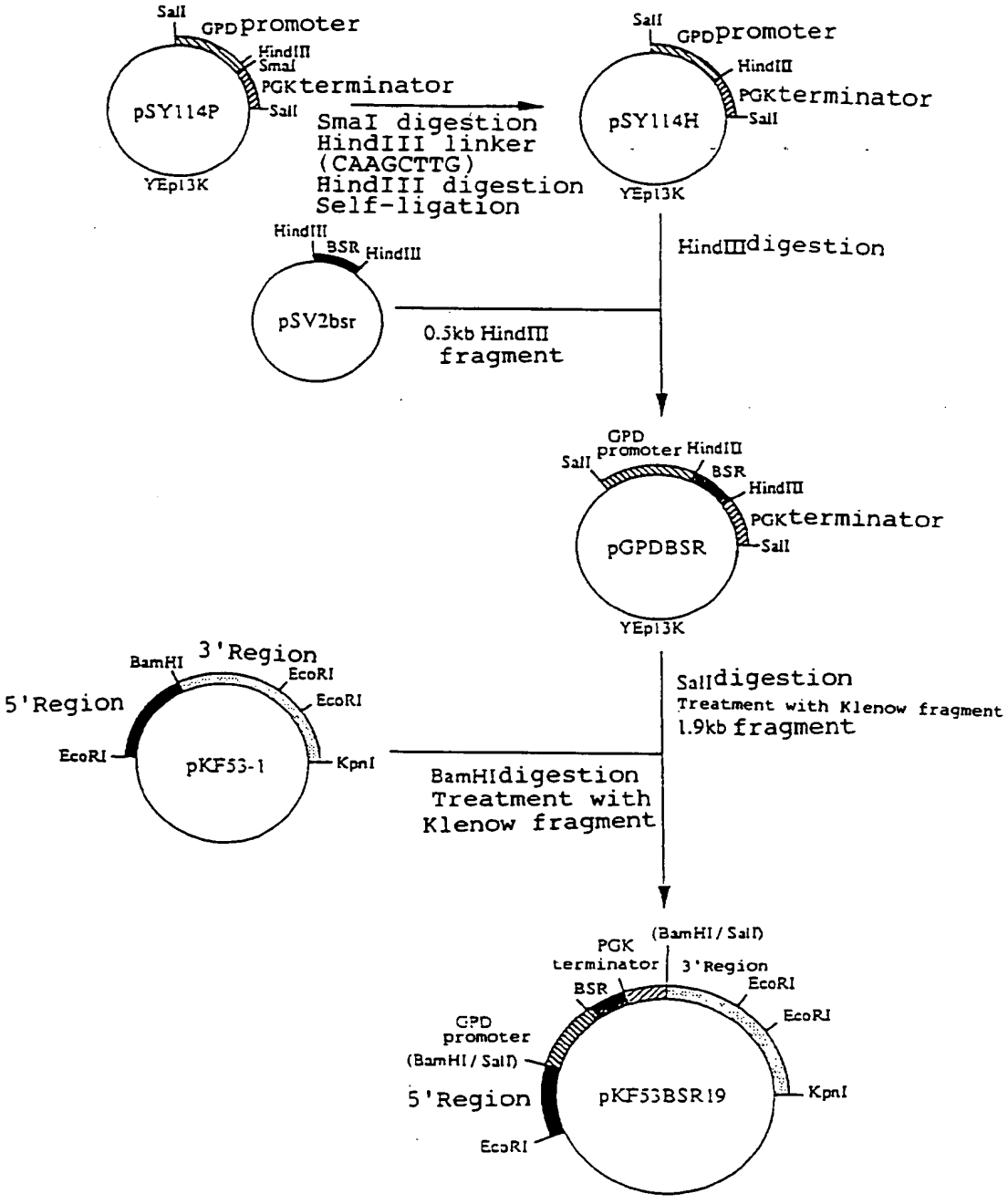
Concept Diagram of Construction of Lg-Sc-Chimeric FLO1 Gene

FIG.6



Construction of a Plasmid for Lg-FLO1 Disruption

FIG.7



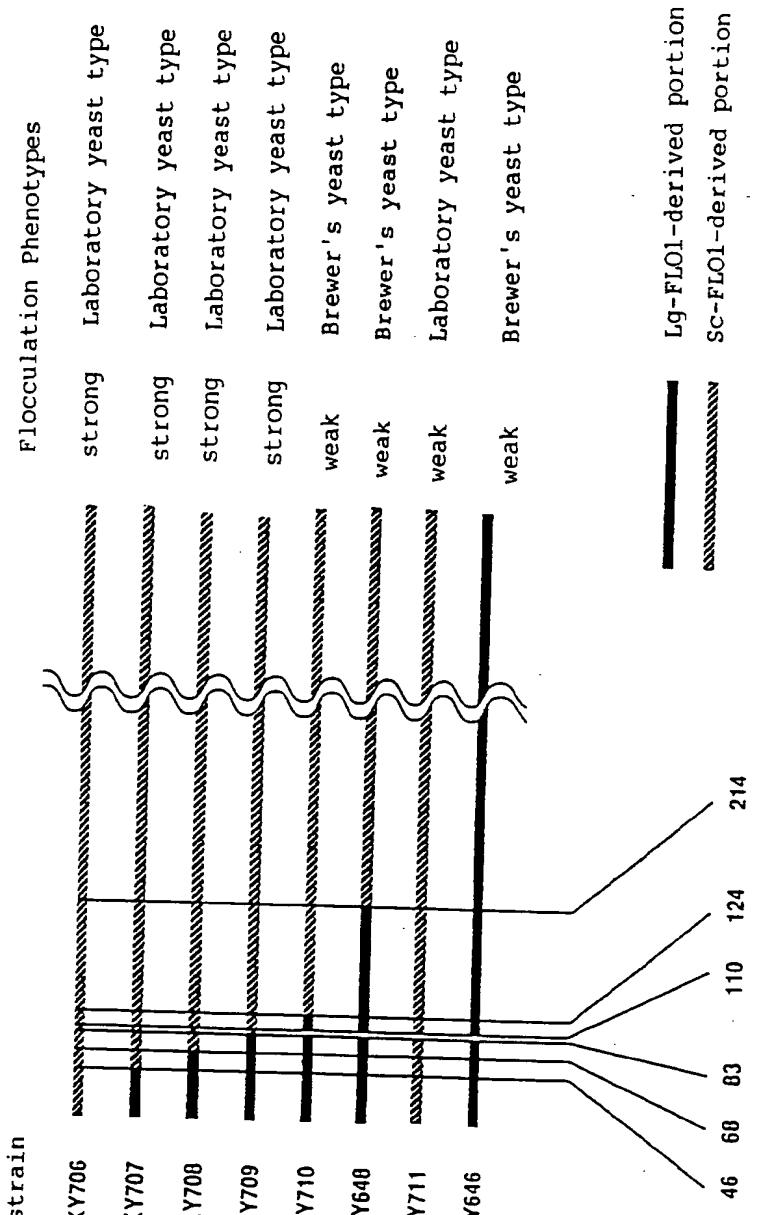
Construction of a Plasmid for Lg-FLO1 Disruption (continued)

FIG.8

```
                10        20        30        40        50        60
Lg-FLO1         MTIAHHCIFLVILAFLELLNVASGSTQACLPVGSRKNGMNVNFYKYSLQDSTTYSDPQYM
                X:..:...::.....:.: .:::::.:::::.: ::.:::.:::.:::.::.::::..::
Sc-FLO1         MTMPHRYMFLAVFTLLALTSVASGATEACLPAGQRKSGMNINFYQYSLKDSSTYSNAAYM

                70        80        90        100       110       120
Lg-FLO1         AYKYSDTKKLGSVSGQTHLSIYY----------------------------GPNTAFWNTA
                :: :...:::::.:::..:: :                              :. ... ..:
Sc-FLO1         AYGYASKTKLGSVGGQTDISIDYNIPCVSSSGTFPCPQEDSYGNWGCKGMGACSNSQGIA

                130       140       150       160       170       180
Lg-FLO1         SWSSDLFGFYTTPTNVTVEMTGYFLPPQTGSYTFKFATVDDSAILSVGGSIAFECCAQEQ
                 ::.:::::::::::::.:::::::::::::::::::::::::::::::..::.::::.:
Sc-FLO1         YWSTDLFGFYTTPTNVTLEMTGYFLPPQTGSYTFKFATVDDSAILSVGGATAFNCCAQQQ

                190       200       210       220       230       240
Lg-FLO1         PPITSTDFTINGIKPWDAAAPTDIKGSTYMYAGYYYPIKIVYSNAKVLARLPVSVVLPDG
                :::::::.:::.:::::::... :..:.:..:::::::::.:.:::::    . ::.::.:::X
Sc-FLO1         PPITSTNFTIDGIKPWGGSLPPNIEGTVYMYAGYYYPMKVVYSNAVSWGTLPISVTLPDG
```

Comparison of Deduced Amino Acid Sequences
for N-Terminal Regions of Lg-*FLO1* and Sc-*FLO1*

FIG.9



Flocculation Phenotypes in Strains with Various Modified *FLO1* Genes

Various modified *FLO1* Genes were ligated in the downstream of the *GAL1* gene and introduced into *FLO1*-disrupted laboratory yeast. Then, flocculating property was evaluated. In flocculation phenotypes, "strong" represents the extent of flocculation as seen in laboratory yeast and "weak" the extent of flocculation as seen in brewer's yeast. With respect to inhibition by sugars, flocculation inhibited by mannose but not inhibited by glucose is expressed as "laboratory yeast type" and flocculation inhibited by either mannose or glucose is expressed as "brewer's yeast type". The figures given at the bottom line indicate the amino acid numbers counted from the N-terminal. When compared to Sc-*FLO1* , Lg-*FLO1* has a deletion of amino acids from position 84 to position 110, but the above amino acid numbers counted from the N-terminal have been converted to the numbers based on Sc-*FLO1*.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/00183

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl[6] C12N15/11, C12N15/31, N12N15/62, C12P21/02, C12N1/19, C07K14/395, C12C11/02 // C12N15/63, (C12N15/31, C12R1:865),
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int. Cl[6] C12N1/00-3/00, C12N15/00-15/90, C12P21/00-21/06, C07K14/39-14/40, C12C11/00-11/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS PREVIEWS, WPI, WPI/L, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TEUNISSEN A.W.R.H. et al. "SEQUENCE OF THE OPEN READING FRAME OF THE FLO1 GENE FROM SACCHAROMYCES CEREVISIAE", YEAST, (1993), Vol. 9, p. 423-427	1 - 21
A	WATARI, J. et al. "CONSTRUCTION OF FLOCCULENT BREWER'S YEAST BY CHROMOSOMAL INTEGRATION OF THE YEAST FLOCCULATION GENE FLO1", J. Inst. Brew., (1994), Vol. 100, p. 73-77	1 - 21
P	TEUNISSEN A.W.R.H. et al. "THE DOMINANT FLOCCULATION GENES OF SACCHAROMYCES CEREVISIAE CONSTITUTE A NEW SUBTELOMERIC GENE FAMILY", YEAST, (1995), Vol. 11, p. 1001-1013	1 - 21
P	MURAKAMI Y. "ANALYSIS OF THE NUCLEOTIDE SEQUENCE OF CHROMOSOME VI FROM SACCHAROMYCES CEREVISIAE" NATURE GENETICS, (1995), Vol. 10, p. 261-268	1 - 21
A	YAMASHITA I. "MATING SIGNALS CONTROL	1 - 21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
May 7, 1996 (07. 05. 96)	May 21, 1996 (21. 05. 96)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/00183

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	EXPRESSION OF BOTH STARCH FERMENTATION GENES AND A NOVEL FLOCCULATION GENE FLO8 IN THE YEAST SACCHAROMYCES" AGRIC. BIOL. CHEM., (1983), Vol. 47, No. 12, p. 2889-2896	1 - 21
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INTERNATIONAL SEARCH REPORT

International application No.
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A. (Continuation) CLASSIFICATION OF SUBJECT MATTER

(C12P21/02, C12R1:865), (C12N1/19, C12R1:865)

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